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(57) Abstract

The present invention relates to the use of growth hormone (GH) secretagogue compound or GH and Adrenocorticotropic hormone (ACTH) secretagogue compound for the manufacture of a medicament for treating cardiac failure or related vascular dysfunction.

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USE OF GROWTH HORMONE SECRETAGOGUE COMPOUND FOR TREATING VASCULAR DYSFUNCTION CARDIAC FAILURE OR RELATED

1. Introduction

The present invention relates to the use of growth hormone (GH) secretagogue compound or GH and Adrenocorticotropic hormone (ACTH) secretagogue compound for the manufacture of a medicament for treating cardiac failure or related vascular dysfunction.

Experimental evidence points to a role of growth hormone (GH) in cardiac physiology. In fact, patients with treated hypopituitarism but without any specific GH replenishment, had an increased mortality from cardiovascular disease, especially myocardial infarction and cardiac failure (8). More recently, in young adults with congenital GH deficiency, a reduction of left ventricular mass and an impairment of the systolic function was found (9). In these patients, GH administration for 6 months increased left ventricular mass and function (Amato, G., C. et al J. Clin. Endocrinol. Metab. 77,1671, 1973). In a recent paper from our group (4), heart preparations from GH deficient rats undergoing low-flow ischemia and reperfusion, were more sensitive to ischemic damage than heart preparations from control rats. GH replacement therapy in these animals reverted heart abnormalities.

Aging has been shown to alter the spectrum of physiological and biochemical properties of the myocardium, including force production, excitation-contraction coupling, substrate utilization and mithocondrial oxidative capacity (1). However, new insights in myocardial-reperfusion injury indicate that aged rats, besides a reduction of the myocardial antioxidant defense mechanisms (2), are affected by alteration of calcium handling in cardiac cells (3). In fact, abnormalities of regulation/modulation mechanisms normally involved in the restriction of calcium oscillation between sarcoplasmic reticulum and cytoplasm are associated with strong impairment of cardiac mechanics.

Recently, it has been shown (4) that growth hormone (GH)-deficiency, induced experimentally in young male rats, is responsible for a marked aggravation of the ischemic damage in hearts subjected to global flow limitation and reperfusion. This aggravation, characterized by a significant increase of ventricular contracture during ischemia and an impaired contractility at reperfusion, is likely related to a defective function of growth hormone (GH) / insulin-like growth factor I (IGF-I) axis. This has here been shown to be completely counteracted by a GH replacement therapy or by administration of hexarelin (5), a novel hexapeptide endowed with a strong GH-releasing activity (6,7).

In this vein, experimental and clinical evidence have already established the pivotal role GH exerts in cardiac pathophysiology (8). Hypopituitary patients given hormone replacement therapy, except for any GH-substitution, had an increased mortality rate from cardiovascular diseases, such as myocardial infarction and cardiac failure (9).

GH secretion and its biological effects decline with aging in both experimental animals and humans (10) (11).

It is known that GHRP (Growth hormone releasing peptide)receptors occur in different tissues and that the binding between GHRPs and the receptor has a high activity especially in heart.

However, the binding as such, does not give enough evidence for an effective treatment of the tissue.

We have now investigate if a peptide with GH releasing activity, here hexarelin, is capable to reverse cardiac dysfunction in a GH-deficiency rat model and the endothelium-dependent relaxing function of coronary arteries and aorta. (Example 1) We have also investigated the protective action of GH against postischemic myocardial dysfunction in hearts from *ex-vivo* treated senescent rats and compared the action of GH with the simultaneous evaluation of the action of a growth hormone (GH) secretagogue compound, here exemplified with hexarelin (Example 2).

Figures

Fig. 1. Cardiac function during moderate ischemia in isovolumic left heart preparations. Example 1.

Fig. 2. Cardiac function during moderate ischemia in isovolumic left heart preparations. Each point of the curves is the mean value of 10 experiments. Example 1.

Fig.3. LVDP. Example 1.

Fig 4. Rate of formation of 6-keto-PGF1a. Example 1.

Fig 5. Vasopressor activity of agiotensin. Example 1.

Fig 6 - Left ventricular pressure (LVP) during postischemic reperfusion in heart preparations from saline- or hexarelin-treated rats. Example 2.

Fig. 7 - Left ventricular developed pressure (LVDP) and coronary perfusion pressure (CPP) in isovolumic left heart preparations. Example 2.

Fig. 8 - Creatine kinase (CK) release profile in ischemic and reperfusion conditions of old rat hearts. Example 2.

Fig. 9 - Rate of release of 6-keto-$PGF_{1\alpha}$ in perfusates of isovolumic left heart preparations. Example 2.

The invention

The attached claims define the present invention.

The finding that a growth hormone (GH) secretagogue compound or a GH and Adrenocorticotropic hormone (ACTH) secretagogue compound has a direct effect on heart is a novel finding, which has not been disclosed earlier and which must be regarded as surprising and of utmost importance.

Growth hormone (GH) secretagogue compounds and GH and Adrenocorticotropic hormone (ACTH) secretagogue compounds include peptides, non-peptides and peptoids. (See E Ghigo et al, J. of Clin. Endocrinology and metabolism, Vol 82, No 8, 1997). This group of compounds do not include natural growth hormone releasing compounds (GHRH/GRF). The claimed compounds are functioning at least partially via the Growth hormone releasing peptide (GHRP) mechanisms.

By GHRP are meant peptidyl GH secretagogue synthetic, non-natural molecules with strong GH- and slight ACTH/Cortisol-releasing effect.

By the expression " related vascular dysfunction" is meant all vascular lesions occurring during cardiac failure.

The protecting activity of the studied compound observed on impaired heart contractility is also related to its effect on endothelium functions. The endothelium elaborates a panoply of proteins, prostanoids and other paracrine substances to maintain a delicate balance between vasoconstriction and vasodilation. A damage of endothelium-dependent vasoconstriction mechanism(s) regulated by nitric oxide (NO) and prostacyclin (PGI2) formed by endothelial cells may initiate and contribute to different pathological states, including hypertension, vasopasm and atherosclerosis.

The heart contractility can be seen in figures 1, the upper panel in figure 2 and figure 3.

The endothelium functions can be seen in figure 2 lower panel and figures 4 and 5.

In the expression " GH-deficiency patients" are patients included whose GH and IGF-I response to spontaneous, physiological and pharmacological tests are GH deficient-like.

The invention is illustrated by the use of hexarelin.

Hexarelin is a low molecular weight peptide with six amino acids:

His - Trp - Ala - Trp - Phe - Lys

in which Trp at position 2 is D-2 methyl-Trp, Phe is D-Phe and Lys is Lys-NH_2.

Hexarelin is a synthetic growth hormone-releasing peptide, shown to produce a substantial increase of growth hormone plasma levels in humans (Imbimbo et al, 1994; Ghigo et al., 1994). The compound is disclosed in the patent application WO 91/18061.

The claims or the illustrating examples do not limit the spirit of the invention.

EXAMPLES

Drugs

The following drugs were used: hexarelin and biosynthetic human growth hormone (Pharmacia, Stockholm, Sweden); angiotensin II (Sigma Chem. Co., MA, USA); multiprime DNA labeling system (Rediprime; Amersham, Little Chalfont, UK); kit for 6-keto-$PGF_{1\alpha}$ determination (Cayman, Chemical Company, Ann Arbor, MI); kit for creatine kinase determination (Boehringer-Mannheim, Germany).

Statistical analysis

Differences of data among groups in individual experiments were analyzed for statistical significance by one-way analysis of variance (ANOVA) and Student's t-test (two-tailed) for unpaired samples. A value of $P<0.05$ was considered significant. The area under the curve (AUC) was assessed using a computerized program Microcal Origin.

Example 1

1.1. Materials and methods

1.1.1 Animals

Pregnant Sprague-Dawley rats (Charles River, Calco, Italy) were purchased and housed under controlled conditions (22±2 °C, 65% humidity and artificial light from 06.00 to 20.00 h). After birth all litters were culled to a standard size of 12 pups. At weaning (20 days), male rats were selected randomly assigned to three experimental groups of 10 animals each and treated with: 1, normal rabbit serum (NRS); 2, anti-GHRH-serum (GHRH-Ab or GH-deficients); 3, anti-GHRH-serum + hexarelin (GHRH-Ab + HEXA).

1.1.2. Antiserum to GHRH

The GHM-Ab was prepared by immunising rabbits with a mixture of synthetic rat GHRH (Spiess, J., J. et al, Nature 303, 532) and methylated BSA emulsified in Freund's adjuvant, as previously described (Benoit et al., 1982, Proc.Natl. Acad. Sci. USA 79, 917). The biologic efficacy of the antiserum was assessed at various

levels. The GHRH-Ab has repeatedly been shown to significantly inhibit GH secretion and growth (Wehrenberg, W.B..et al 1984 , Endocrinology 115, 1218.; Wehrenberg, W.B. et al, 1986, Endocrinology 118, 489; Arsenijevic et al., 1989, Endocrinology 124, 3050). In addition, the antiserum was tested for rat GHRH-binding capacity with ^{125}I-labelled rat GHRH. The antiserum dilution required to bind 30% of the tracer was approximately 1:30.000. Characterisation of the antiserum showed that it was directed toward the GHRH carboxyl terminal. It cross-reacted with synthetic human, bovine and porcine GHRH by less than 4%, and the dose-response curves were not paralleled with rat GHRH. The antiserum did not cross-react with peptides that have considerable sequence homology with GHRH, including secretin, glucagon, vasoactive intestinal peptide, gastrin motilin, bradykinin and angiotensin.

1.1.3. Treatments

Rats were treated every other day by s.c. administration of the anti-GHRH serum (250 µl/rat) or isovolumetric amounts of normal rabbit serum from postnatal day 20 to 40. A group of anti-GHRH serum treated rats was given in addition hexarelin (80 µg/kg s.c., bid) from postnatal day 25 to 40 (15 days). At 41 days of life, about 14 h after the last injection of hexarelin, rats were killed by decapitation. Pituitaries were removed, immediately frozen on dry ice, and stored at -20°C until used. Blood was collected into EDTA-containing tubes and plasma was separated and stored at -20 °C for insulin-like growth factor I (IGF-I) determination.

1.1.4. Pituitary GHmRNA and plasma insulin-like growth factor I (IGF-I) levels

For the evaluation of GH mRNA levels, 10 pituitaries from each experimental group were collected in pools of two samples (5 pools per experimental group). Total RNA was obtained by single-step acid guanidium-phenol-chloroform extraction (14 and Sacchi, 1987). Total RNA samples (20 mg/sample) were electrophoresed on 1.2 % formaldehyde-agarose gel and transferred to a nitro-

cellulose membrane at room temperature for 24 h in 10 times saline sodium citrate (SSC) (1 x SSC = 0.1 M sodium chloride/0.01 M sodium citrate). Filters were hybridised with a rat GH cDNA sequence (13 and 15) labelled by the Multiprime DNA labelling system with α [^{32}P] dCTP to a specific activity of 1x10^9 dpm/µg DNA. Hybridisation conditions were as previously reported (13 and 15). Quantification of the hybridisation signal was performed on a scanning densitometer (LKB XL Laser Densitometer, LKB, Uppsala, Sweden). Pituitary GH mRNA levels were expressed as percent value of normal rabbit serum-treated rats.

Plasma IGF-I levels were evaluated by a homologous radioimmunoassay in plasma extracted with 12.5% of 2N HCl plus 87.5% ethanol using reagents provided by the National Hormone and Pituitary Program (NHOP). The sensitivity of the assay was 100 pg/ml; intra- and interassay variation was less than 10%. The IGF-I plasma levels of 10 rats for each experimental group were determined.

1.1.5. Perfused rat heart preparations

As previously described (17), the hearts from the three experimental groups were rapidly removed and perfused retrogradely through the aorta with Krebs-Henseleit solution (37°C) of the following composition (in mM): NaCl 118, KCl 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 5.5. The solution was gassed with a mixture of 95% 0_2 + 5% $C0_2$ and, after a 30 min equilibration period, the pH of the heart perfusate was 7.4. Left ventricular pressure (LVP) was measured by a polyethylene catheter (with a small latex balloon on the top) inserted in the left ventricle cavity. The balloon was filled slowly with saline with a micrometer syringe until left ventricular end-diastolic pressure (LVEDP) stabilised in the range of 5 mmHg. Coronary perfusion pressure (CPP) and LVP were monitored with Statham transducers (HP-128OC) connected to a Hewlett-Packard (Waltham, MA, USA) dynograph (HP-7754A). The hearts were electrically paced at a frequency of 300 beats/min with rectangular impulses (1 ms duratio; voltage 10% above threshold) by a Grass stimulator (mod. S-88; Grass Instr., Quincy, MA, USA). The perfusion rate of each heart was adjusted to yield a CPP of 55-60

mmHg with a flow rate of 12 ml/min. Ischemia was induced by reducing the coronary flow to 2 ml/min with a perfusion pressure of 4-6 mmHg. Each heart was reperfused 40 min after the onset of ischemia at the preischemic flow rate (12 ml/min) for another period of 20 min. The vasopressor activity of angiotensin II (1 µg injected as a bolus in the perfusion system) was regularly recorded at the beginning of each experiment.

1.1.6. 6-Keto-PGF $_{1\alpha}$ in heart perfusates

Prostacyclin (PGI_2) generation was measured in the heart perfusates as 6-Keto-$PGF_{1\alpha}$, according to the enzyme immunoassay previously described by (18). Particularly, the concentration of this eicosanoid was determined collecting the heart perfusates for 5 min immediately before flow reduction and during the first 10 min of reperfusion.

1. 2.Results

1.2.1.Growth rate

Starting from day 28, i.e. 8 days after the beginning of treatment with the anti-GHRH serum, rats grew significantly less than normal rabbit serum-treated rats ($P<0.05$); at the end of the experiment, the mean weight of the GH-deficient rats was 13% less ($P<0.01$) than that of control animals. In anti-GHRH serum + hexarelin-treated rats, peptide replacement completely counteracted the growth inhibitory effect of the antiserum and no significant difference between body weight of this group of animals and that of control rats was observed (Table 1). In anti-GHRH serum-treated animals, heart weight was reduced of 14% ($P<0.01$) as compared to control and anti-GHRH serum + hexarelin-treated rats (Table 1). However, the ratio heart weight/body weight was similar in the three experimental groups of animals. This indicates that in the GH-deficient rats the decrease of heart weight was proportional to that of body weight.

1.2.2.Pituitary GH mRNA and plasma IGF-I levels

As shown in Table 2, pituitary GH mRNA and plasma IGF-I levels were reduced of 51.2% ($P<0.01$) and 43.5% ($P<0.01$) respectively in GH-deficient rats as compared to normal rabbit serum-treated animals. Administration of hexarelin to anti-GHRH serum-treated rats restored both pituitary GH mRNA and plasma IGF-I at the level of control animals.

1.2.3.Ischemia-reperfusion in isolated-rat hearts

When the rate of perfusion of paced isovolumic left heart preparations, obtained from normal rabbit serum-treated rats (control) was reduced from 12 ml/min to 2 ml/min, peak left ventricular systolic pressure and maximum left ventricular dP/dt ($LVdP/dt_{max}$) declined rapidly. At the same time, the phasic contracfility of the hearts slowed until complete ventricular arrest was achieved. Afterwards, only a minimal elevation of LVEDP was recorded during the ischemia and reperfusion periods (Fig. 1, 2). At the reperfusion a substantial recovery of cardiac contractility (65%; $P<0.01$) with a prompt regaining of the electrical pacing was recorded. In these hearts CPP values were only minimally affected, indicating that a modest increase in coronary resistance as well as an ischemic damage of very low degree was occurred (Fig. 1, 2).

When the ischemia-reperfusion experiments were repeated in hearts excised from GH-deficient rats, a worsening of the ischemic damage, as compared to control hearts, was observed (Fig. 1, 2). In these cases the ventricular contraction (rise in LVEDP) at the end of the ischemic period was 7 times higher ($P<0.001$) than that observed in the corresponding control preparations (Fig. 1, 2). During reperfusion, due to a marked elevation of LVEDP, a poor recovery of mechanical activity, associated with persistent rhythm disturbances was monitored. Moreover, at the end of 20 min-reperfusion, CPP values were still significantly elevated (+225 % vs controls; $P<0.001$) and this was in part due to a certain degree of heart stiffness (Fig. 1, 2).

When the hearts from anti-GHRH serum + hexarelin-treated rats were subjected to reduction of perfusion flow and reperfusion, the trend of the ischemic damage was similar to that observed in control hearts. In fact, the ventricular contracture was

markedly reduced, being at the end of the ischemic phase only two times higher ($P<0.01$) than that obtained in control hearts. Moreover, at the reperfusion, the prompt appearance of the electrical pacing favoured a complete recovery of heart contractility (Fig. 1, 2). At the same time, CPP values were very little affected, being at the end of reperfusion statistically indistinguishable from those of control -hearts (Fig.1, 2). It is interesting to underline that, when the LV-developed pressure (peak LV systolic pressure minus LVEDP) was evaluated during the reperfusion period, the hearts obtained from GH deficient rats treated with hexarelin provided the most favourable results even in comparison to that of control rats (Fig. 3).

1.2.4.6-Keto-PGF$_{1\alpha}$ generation in perfused rat hearts

The concentration in the heart perfusates of 6-Keto-PGF$_{1\alpha}$ during the 5 min preceding the ischemic period and during the first 10 min of reperfusion is shown in Fig. 4. Considering the results with hearts obtained from GH-deficient rats, the rate of formation of this eicosanoid was significantly diminished as compared to control hearts. In fact, during the preischemic period -and reperfusion, the reduction was in the range of 50% ($P<0.01$). On the contrary, hearts obtained from GH-deficient rats treated with hexarelin showed a rate of formation of 6-Keto-PGF$_{1\alpha}$ which was not statistically different ($P>0.05$) from that observed in control hearts.

1.2.5 Vasopressor activity of angiotensin II

As shown in Fig. 1 and 5, bolus injections of angiotensin-II (1 μg) in the perfusion system of hearts excised from GH-deficient rats induced a vasopressor activity which was markedly increased (297%; $P<0.001$) as compared with control hearts. Furthermore using hearts obtained from GH-deficient rats treated with hexarelin the response of the coronary vasculature to angiotensin-II was not statistically different ($P>0.05$) from that monitored in control hearts.

Legends to Figures related to Example 1.

Fig. 1. Cardiac function during moderate ischemia and reperfusion in isovolumic left heart preparations of the rat electrically driven.

NRS: the heart was excised from a rat treated with normal rabbit serum (control); GHRH-Ab: the heart was excised from a rat treated with anti-GHRH senim (GH-deficient); GHRH-Ab +HEXA: the heart was excised from a rat treated with antiGHRH serum + hexarelin. At the arrow: angiotensin II (AII) was injected as a bolus (1 μg) in the perfusion system. LVP = left ventricular pressure; CPP = coronary perfusion pressure; LV dP/dt_{max} = first derivative of LVP.

Fig. 2. Cardiac function during moderate ischemia and reperfusion in isovolumic left heart preparations of the rat electrically driven. LVEDP = left ventricular end-diastolic pressure; CPP = coronary perfusion pressure. The legend as in Fig. 1. Each point of the curves is the mean value of 10 experiments and vertical bars S.E.M.

AUC (area under the curve) values related to LVEDP: NRS = 172±18; GHRH-Ab = 1185±93 [b]; GHRH-Ab + HEXA = 364±25 [a]; Differences versus NRS: [a] $P<0.05$; [b] $P<0.001$.

The AUC was evaluated by the trapezoid method: in ordinate, LVEDP in mmHg; in abscissa, time from 0 to 60 min.

Fig. 3. Left ventricular developed pressure (LVDP = peak left ventricular systolic pressure minus LVEDP) in isovolumic left heart preparations of the rat electrically paced. Each point of the curve is the mean value of 10 experiments and vertical bars S.E.M. The legend as in Fig. 1.

AUC (area under the curve) values related to LVDP: NRS = 418±36; GHRH-Ab = 138±16 [b]; GHRH-Ab + HEXA = 589±41 [a] Differences versus NRS: [a] $P<0.05$; [b] $P<0.001$.

The AUC was evaluated by the trapezoid method: in ordinate, LVEDP in mmHg; in abscissa, time from 40 to 60 min.

Fig. 4. Rate of formation of 6-Keto-$PGF_{1\alpha}$ in isovolumic left heart preparations of the rat electrically paced. The legend as in Fig. 1. Perfusates were collected for 5 min before reduction of the flow rate (preischemia) and during the first 10 min of

reperfusion. Each columns represent the mean values of 10 hearts and vertical bars S.E.M. [a] P<0.01 versus NRS and GHRH-Ab + HEXA.

Fig. 5. Vasopressor activity of angiotensin II (1 µg bolus) injected in the perfusion system of isovolumic left heart preparations of the rat electrically paced. The legend as in Fig. 1. Each column represent the mean value of 10 hearts and vertical bars S.E-M. CPP = coronary perfusion pressure during the preischemic period. [a] P<0.001 versus NRS and GHRH-Ab + HEXA.

1.3 Discussion of Example 1.

In a recent study from our group (4), rats passively immunised against GHRH, a suitable model of selective GH deficiency (12; Shakutsui et al., 1989: Acta Paediat. Scand. Suppl. 349, 101; 13 and 15), exhibited clear signs of cardiac dysfunction, consisting of an exacerbation of ischemic tissue damage during low-flow ischemia and reperfusion, with increased coronary artery resistance upon reperfusion. These heart abnormalities were reverted to normal by "ex vivo" replacement therapy with GH (4). In the present study, we tested the possibility of restoring cardiac function in anti-GHRH serum-treated rats by administration of hexarelin, a synthetic hexapeptide endowed with a potent GH-releasing activity (4).

The anti-GHRH serum-treated rats used were truly GH-deficient as shown by decreased growth rate, pituitary GH mRNA and plasma IGF-1 levels, all features reported in previous studies (Arsenijevic et al., 1989, Endocrinology 124, 3050; ; Shakutsui et al., 1989: Acta Paediat. Scand. Suppl. 349, 101; 17). In these rats, somatotropic function was restored by hexarelin replacement as proved by normalisation of all biological markers investigated. Restoration of GH mRNA levels in anti-GHRH serum young adult male rats at the same doses used in these experiments was already reported by Torsello, A., M. et al, 1996, Neuroendocrinology *(in press)*.

The mechanism(s) underlying the action of hexarelin is not fully understood; this peptide may modulate GH secretion by acting directly on the pituitary (Pong et al., 1991, Proceeding of the 73rd Meeting of the Endocrine Society, p.88; Smith,

R.G., K.et al, 1993, Science 260, 1640) or at hypothalamic level by modulating the release of somatostatin (Clark, R.G.,et al, 1989, J. Neuroendocrinol. 1, 249; Bowers et al., 1991, Endocrinology 128, 2027) and/or GHRH (Bercu et al., 1982, Endocrinology 130,2579; Clark et al., 1989, Neuroscience, 53, 303) and/or some unknown factor (Bowers e t al., 1991, Endocrinology 128, 2027). The results, indicating a worsening of the ischemic damage in hearts from GH-deficient rats are not easily explainable. The remarkable increase in ventricular contracture observed upon flow reduction in these hearts could be due to a lack of a possible "modulatory role" of GH and/or IGF-I in membrane ion permeability leading to Ca^{++} accumulation in myocardiocytes. In fact, a good correlation between the cardiomechanical changes typical of ischemia and Ca^{++} accumulation in the mitochondria cellular fraction of myocardiocytes has been already reported (Henry et al. 1977, Am. J. Physiol. 233, H677).

Calcium plays a key role on the energy metabolism of cardiac muscle, and disturbancy in the amount of distribution of intracellular Ca^{++} may affect the energetics of myocardial cells (Gergely, J. 1976, Foderation Proc. 35. 1283). Excess of intracellular Ca^{++} could enhance ATP utilisation and simultaneously diminish its production: the ensuing limitation of ATP availability may thus induce decrease myocardial compliance which favours ventricular contracture with a poor recovery of contractility at reperfusion. Experiments are now in progress to establish directly the capacity of both hexarelin and GH to reduce Ca^{++} overloading during myocardial ischemia. Another point emerging from these studies is that hexarelin per se, or via GH release, caused an improvement of LV-developed pressure during reperfusion in heart from GH-deficient rats, which was significantly superior to that of normal rabbit serum treated rats. This phenomenon is again difficult to explain. However, it is tempting to speculate that the accumulation of Ca^{++} in miocytes during myocardial ischemia may have activated a constitutive Ca^{++}/calmodulin sensitive nitric-oxide synthese (cNOS) with an increase production of nitric oxide (NO) which in turn may have contributed to the maintenance of a depressed heart contractility. In this regard, the recognition of cardiac myocyte cNOS and the consequent implication of increased NO generation in the negative inotropic effect of heart contractility has been reported

by various Authors (Finkel et al., 1995, J Pharmacol Exper. Ther, 272, 945: Kelly, R.A et al. Circulation Research 79, 363, 1996). To test this hypothesis, experiments have been designed to investigate whether L-monomethyl-arginine, a well known inhibitor of cNOS activity, may improve heart contractility during reperfusion in heart from GH deficient rats subjected to low-flow ischemia.

Another interesting feature of GH-deficiency emerging from these experiments is the remarkable increase in sensitivity of coronary vasculature to the vasopressor activity of angiotensin II, an event completely reverted by hexarelin. These data seem to suggest that lack of GH in the rat may have caused an impairment of endothelium-dependent relaxing function of the coronary bed. This hypothesis is reinforced by the observation that the rate of formation of PGI_2 is significantly reduced in hearts from GH-deficient animals. This eicosanoid is generated in endothelial cells lining the vasculature and participates, with NO, in the regulation of blood pressure and in the moderation of vasoconstrictor's activity (36).

Preliminary results obtained with specimen of thoracic aorta excised from GH-deficient rats indicate that the alteration of endothelial-cell function is a more general phenomenon which is not limited to coronary vasculature.

Taken together, the present findings not only confirm that GH-deficiency in rats may be responsible of abnormalities of the cardiac tissues which become more sensitive to ischemia-reperfusion, but also that hexarelin, regardless of the mechanisms involved, mimics the protective activity of GH already reported in GH-deficient rats.

1.4 Conclusion of Example 1.

The ability of hexarelin, a recently synthetized hexapeptide with a remarkable GH-releasing activity, to reverse the worsening of cardiac dysfunction in GH-deficient animals was studied in young male rats made GH deficient by administration of an anti-GH-releasing hormone serum (GHRH-Ab) from 20 to 40 days of life. Heart preparations from GHRH-Ab-treated rats, subjected to lowflow ischemia and reperfusion, showed: 1) a progressive increase of left ventricular

end-diastolic pressure (LVEDP) during the ischemic period and a poor recovery of mechanical activity at reperfusion with a significative decrease of the left ventricular (LV)-developed pressure as compared to control hearts; 2) a decreased rate of formation of 6-Keto-$PGF_{1\alpha}$, the stable metabolite of prostacyclin, in perfusates of both preischemic and reperfusion periods; 3) an increased vasopressor activity of angiotensin II on the coronary vasculature. Hexarelin (80 µg/kg, bid, sc), administered for 15 days (from 25th postnatal day) to GHRH-Ab-treated rats reversed these signs of cardiac dysfunction. In particular, in heart preparations from GHRH-Ab + hexarelin-treated rats the trend of the ischemic damage was similar to that observed in control hearts and, during reperfusion, the (LV)-developed pressure was increased over the control values. In this set of experiments, the rate of formation of 6-Keto-$PGF_{1\alpha}$ and the vasopressor activity of angiotensin II were both reverted to control levels. These results indicate that GH-deficiency in rats is responsible for an impairment of the cardiac function which is associated with a damage of the endothelium lining the coronary vasculature. These alterations are fully reverted by an "'in vivo" treatment with hexarelin.

Table 1. Growth rate of young male rats of 41 days of age.

WEIGHT	NRS	GHRH-Ab	GHRH-Ab + HEXARELIN
BODY (g)	193.1 ± 2-2	168.2 ± 2.1[a]	192.8 ± 1.8
HEART (mg)	1475 ± 10.1	1295 ± 9.0[a]	1480 ± 12-8
HEARTIBODY (mg/g)	7.63	7.69	7.67

Each figure is a mean value ± S.E.M. of 10 determinations.

NRS: rats treated with normal rabbit serum; GHRH-Ab: rats treated with anti-GHRH serum

[a]P<0.01 versus NRS and GHM-Ab + HEXARELIN

Table 2. Markers of somatotropic function of young male rats of 41 days of age.

TREATMENT	PITUITARY GH (m RNA %)	PLASMA IGF-I (ng/ml)
NRS	100	165 ± 49
GHRH-Ab	-51.2 ± 1.7[a]	93.4 ± 2.4[a]
GHRH-AB + HEXARELIN	-7.1±6.1	157.3 ± 2.4

Figures related to GH mRNA are the mean values ± S.E.M. of 5 determinations. Figures related to plasma IGF-I are the mean values ± S.E.M. of 10 determinations.

[a]P<0.01 versus NRS and GHRH-Ab + HEXARELIN

Example 2.

2.1 Materials and methods

2.1.1.Animals and treatments

Twenty-four-month old male rats of Sprague-Dawley strain in good health status, body weight 850 ± 70 g, were purchased (Harlan Nossan, Correzzana, MI, Italy) and housed under controlled conditions (22 ± 2°C, 65% humidity, artificial light from 06.00 to 20.00) with free access to food and water. They were randomly assigned to three experimental groups and treated subcutaneously with: *a*, 1 ml/kg saline (controls, n=10); *b*, biosynthetic human growth hormone (GH, n=6); *c*, hexarelin (HEXA, n=9).

Hexarelin (His-D-2-Me-Trp-Ala-Trp-D-Phe-Lys-NH_2) and GH were given to rats at the dose of 80 μg/kg and 0.4 μg/g bid respectively, for 21 days. The dose of hexarelin or GH was chosen on the basis of previous results showing their adequacy to restore somatotropic function in neuroendocrine (12, 13) and cardiovascular studies (4). Animals were killed by cervical dislocation 14 h after the last injection. Pituitaries were removed, immediately frozen on dry ice and stored at -20°C until used for determination of GH mRNA levels. Blood was

collected into EDTA-containing tubes and plasma was separated and stored at -20°C for IGF-I determination. The hearts were isolated and used for ischemia and reperfusion experiments.

2.1.2.Pituitary GH mRNA and plasma IGF-I levels.

Total RNA was isolated from each pituitary by the single-step acid guanidium-phenol-chloroform extraction (14). Total RNA samples (20 µg/sample) were electrophoresed on 1.2% formaldehyde-agarose gel and transferred to nylon membranes (Hybond N, Amersham. Little Chalfont, UK). The membranes were hybridized with a rat cDNA sequence (13,15) labeled by random primer with [α-^{32}P] dCTP to a specific activity of 10^9 dpm/µg DNA. Hybridization conditions were as previously reported (13, 15). Quantification of the hybridization signal was performed on a scanning densitometer (LKB XL Laser Densitometer, LKB, Uppsala, Sweden). Pituitary GH mRNA levels were expressed as percentage of controls values.

Plasma IGF-I levels were evaluated by a homologous radioimmunoassay in plasma after acid-ethanol extraction according to the method described by Daughaday (16). The reagents were provided by the National Hormone and Pituitary Program. The sensitivity of the assay was 100 pg/ml; intra- and inter-assay variation was less than 10%. The IGF-I plasma levels of 6-10 rats for each experimental group were determined and expressed in ng/ml.

2.1.3.Perfused rat heart preparations

Hearts from the three experimental groups of rats were perfused retrogradely at 37°C through the aorta following a method described by Berti et al. (17). The perfusion medium contained (in mM): NaCl 118, KCl 2.8, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 5.5. After a period of equilibration with 5% CO_2 and 95% O_2 gas mixture, the pH of the perfusate was 7.35 and the perfusion was maintained at 15 ml/min with a roller pump (Minipuls 3, Gilson Villiers, le Bel, France). Left ventricular pressure (LVP) and coronary perfusion pressure (CPP) were recorded using a HP-1280C pressure transducers (Hewlett-Packard, Waltham, MA, USA). LVP was obtained by inserting a small latex

balloon filled with saline through the left atrium. Left ventricular end diastolic pressure (LVEDP) was stabilized to 4-5 mmHg, whereas CPP was maintained at 65-70 mmHg. All these variables were displayed on Hewlett-Packard dynograph (HP-7754A). The hearts were electrically paced at the frequency of 300 beats/min with rectangular impulses (1 msec duration, voltage 10% above threshold) by a Grass stimulator (model S-88, Grass Instruments, Quincy, Mass, USA).

A moderate ischemia (stunning) was induced by global reduction of the perfusion flow to 1 ml/min for a period of 20 min. A normal flow rate (15 ml/min) was then restored and reperfusion continued for 30 min. Left ventricular developed pressure (LVDP = peak left ventricular systolic pressure minus LVEDP) was evaluated during reperfusion. At the beginning of each experiments the activity of angiotensins-II (from 0.25 to 4 μg as bolus in the perfusion system) on coronary vasculature was recorded.

2.1.4. 6-keto-PGF$_{1\alpha}$ in heart perfusates

Prostacyclin (PGI_2) generation by the cardiac tissues was measured in heart perfusates as 6-keto-$PGF_{1\alpha}$ according to the enzyme immunoassay method (detection limit 0.05 ng/ml) of Pradelles et al. (18). The concentration of this stable metabolite was determined collecting the perfusates for 5 min immediately before flow reduction and during the first 10 min of reperfusion. The rate of formation of 6-keto-$PGF_{1\alpha}$ was expressed in ng/min.

2.1.5. Creatine kinase in heart perfusates

The perfusate was collected every 150 sec in an ice-cooled beaker before flow reduction and during reperfusion and the activity of creatine kinase (CK) was evaluated according to the method of Bergmeyer et al. (19). The amount of the enzyme was determined on a spectrophotometer (Lambda 16, Perkin Elmer Italia, Monza, MI, Italy) and expressed as mU/min/g wet tissue.

2.2. Results

2.2.1 Somatotropic function of old male rats

Treatment of 24-month-old male rats with hexarelin or rhGH did not apparently affect the basal somatotropic function, pituitary GH mRNA and plasma IGF-I levels being in the range of values measured in saline-treated controls. During these treatments, rats did not loose weight nor showed any particular sign of toxicity and also systemic blood pressure and heart rate did not change. The heart weight/body weight ratio was in the three experimental groups not statistically different, indicating that neither hexarelin nor GH treatment had increased the cardiac ventricular mass (Table 3).

2.2.2. Ischemia and reperfusion in isolated rat hearts

The global reduction of flow for 20 min (from 15 ml/min to 1 ml/min) in isovolumic left heart preparations obtained from saline-treated rats, induced a clear-cut decrease of left ventricular function associated to a substantial increase in coronary resistance. In fact, the recovery of postischemic LVDP was low and after 30 min of reperfusion only the 37% of the pre-schemic strength of heart contractility was restored; at this time CPP was still 71% over the basal values and the event was not associated with stiffness of the hearts (Fig. 6 and 7).

Furthermore, the partial functional recovery of the hearts during reperfusion was accompanied by a consistent release of CK into the perfusates. In fact, peak concentration of CK was increased 6.6 fold ($P<0.001$) over pre-schemic values and, at the end of reperfusion, was still significantly elevated (182%; $P<0.01$) (Fig. 8).

In contrast to rat heart preparations obtained from saline-treated control rats, there was a striking protective effect against the reperfusion damage in heart preparations from hexarelin-treated rats (Fig. 6 and 7). In fact, already at the beginning of the reperfusion, a regular paced rhythm appeared and the recovery of post-ischemic left ventricular function was in the range of 73% of the pre-schemic strength. After 30 min, LVDP values stabilized at 90% ($P<0.001$) of those recorded during preischemia (Fig. 7). In these preparations CPP values increased only minimally in the first 5 min of reperfusion (19%) and basal values were attained at the end of this period. In keeping with these results, the kinetic profile

of CK released in the effluent was significantly different from that observed in control preparations. At the peak of the concentration, CK was increased only 2 fold ($P<0.01$) with a gradual return toward baseline at the end of reperfusion.

A lower protective activity against reperfusion damage was present in heart preparations obtained from GH-treated rats. In these series of experiments the trend of postischemic left ventricular dysfunction was similar to that present in hearts from control rats, though, at the end of 30 min of reperfusion the LVDP reached 55% ($P<0.01$) of the pre-schemic values (Fig. 7). Furthermore, CPP was increased of 65% ($P<0.01$) over the basal values at the beginning of reperfusion and was still markedly elevated after 30 min (46% increase; $P<0.01$) (Fig. 7). These results were also reflected by a marked increase of CK in the effluent (433% over basal values; $P<0.001$), peaking between 8 and 15 min of reperfusion, and still evident at 30 min (109% increase; $P<0.01$) (Fig. 8).

2.2.3. 6-keto-$PGF_{1\alpha}$ generation in perfused rat hearts and angiotensin II activity

The rate of release of 6-keto-$PGF_{1\alpha}$ in the perfusates of hearts from the three experimental groups was not statistically different (2-2.5 ng/min). As expected, during the first 10 min of reperfusion the generation of the prostacyclin metabolite increased approximately 5 fold (8.5-10 ng/min) in hearts from controls, hexarelin- or GH-treated rats (Fig. 9). This would indicate that the beneficial effect exerted by the two peptides in postischemic left ventricular dysfunction was not related to further stimulation of 6-keto-$PGF_{1\alpha}$ formation by the heart tissues.

Bolus injections of angiotensin II (0.25-4 μg) into heart preparations at the beginning of each experiment induced a dose-related increase in CPP. The dose-response curves of the vasopressor activity of Angiotensin II were not statistically different in the three experimental groups of hearts, thus implying that either hexarelin or GH did not interfere with the endothelium-dependent relaxant function of coronary vasculature (data not shown).

Legends to Figures related to Example 2.

Fig. 6 - Left ventricular pressure (LVP) during postischemic reperfusion in heart preparations from saline- or hexarelin-treated rats.

Fig. 7 - Left ventricular developed pressure (LVDP) and coronary perfusion pressure (CPP) in isovolumic left heart preparations submitted to low flow ischemia and reperfusion from old-rats of the following experimental groups: a, saline (controls, n=10); b, human growth hormone (GH, n=6); c, hexarelin (HEXA, n=9). Each point on the curves depicts mean values and vertical bars standard error of the mean. The area under the curve (AUC) related to LVDP are: a, 765 ± 46; b, 1147 ± 88; c, 2272 ± 66. Statistical differences: c vs. b and a: P <0.01; b vs. a: P<0.05. The AUC related to CPP (increase in mmHg over the pre-schemic values) are: a, 1284 ± 79; b, 1008 ± 47; c, 235 ± 35. Statistical significance: c vs. b and a: P<0.01; b vs. a: P<0.05. AUC was estimated according the trapezoid method: in ordinate, LVDP or CPP in mmHg; in abscissa, time from 20 to 50 min.

Fig. 8 - Creatine kinase (CK) release profile in ischemic and reperfusion conditions of old rat hearts. The area under the curve (AUC) related to CK release during reperfusion are: a, 4454 ± 352; b, 3520 ± 278; c, 278 ± 56. Statistical differences: c vs. a and b: P<0.01; b vs. a: P<0.05.

Fig. 9 - Rate of release of 6-keto-$PGF_{1\alpha}$ in perfusates of isovolumic left heart preparations from old-rats of the three experimental groups. Columns represent mean values and vertical bars standard error of the mean. Perfusates were collected during preischemia (5 min) and reperfusion (first 10 min). Values obtained during preischemia are statistically different from those of reperfusion: P<0.001.

2.3.Discussion of Example 2.

Myocardial ischemia, defined as an imbalance between fractional uptake of oxygen and the rate of cellular oxidation, may have several potential outcomes, especially in senescent hearts which are the ones more prone to this pathological event. Under these circumstances, when ischemia is brief, a transient postischemic ventricular dysfunction may occur and this condition (stunning) reflects many disturbances of cardiomyocytes and insufficient cellular antioxidant activity (2, 20). In the present model of ischemia-reperfusion in hearts from old rats chronically treated with hexarelin, a considerable protection against

mechanical stunning was achieved. It is noteworthy that complete recovery of left ventricular function was present upon reperfusion. Simultaneous blunting of the release of CK in the heart effluents underlined the integrity of myocardial cell membranes and the preservation from the contractile impairment which follows oxygen readmission.

The beneficial effect disclosed by hexarelin in aged rats, under our experimental conditions, was not coupled to any apparent stimulation of the somatotropic function, since the level of pituitary GH mRNA and plasma IGF-I were unchanged. This would indicate, albeit inferentially, that the hexapeptide had a direct myocardial action which was not mediated by GH (see also below). Favoring this view, Grilli et al. (21) and Howard et al. (22) have recently reported that mRNA coding for a receptor related to GH-releasing secretagogues (GHS) is expressed in peripheral organs of male rats, heart included.

We still ignore what kind of intracellular signal transduction is triggered by GHS-receptor activation in peripheral organs, a point which deserves a thorough investigation. However, the striking hexarelin-induced inhibition of reperfusion damage in the isolated hearts calls for a restraint in the increase of cytosolic calcium which follows reperfusion (23, 24). In this context, either the inhibitor of sarcoplasmic reticulum function, ryanodin, or the transsarcolemmal calcium-channel blocker diltiazem, were shown capable to improve recovery of left ventricular developed pressure in rabbit hearts subjected to ischemia and reperfusion (25).

A high vulnerability to moderate ischemia in senescent rat hearts is supported by the increased calcium regulating protein gene expression associated with a strong impairment of contractile function (26).

Another feature of considerable importance of the present studies was the protective effect induced by GH in the heart preparations from senescent rats. Although the improvement of post-ischemic ventricular function was modest and by no means comparable, under our experimental conditions, to that elicited by hexarelin, it is likely attributable to a direct action of the hormone on the heart where receptors for both GH (27) and IGF-I (28, 29) have been identified. Reportedly, the GH-receptor gene is expressed to a greater extent in the

myocardium than in any other tissue (27) and in hypophysectomized rats GH administration increases cardiac IGF-content (30) and induces IGF-I mRNA expression (31). IGF-I itself has a positive inotropic effect on the isolated perfused rat hearts (32) and it limits after myocardial ischemia the reperfusion damage by inhibiting apoptosis and leukocyte-induced cardiac necrosis (33).

In our study, we also investigated the ability of the cardiac tissues to generate 6-keto-$PGF_{1\alpha}$, the stable metabolite of prostacyclin, whose increase during the reperfusion period would contribute, with other biochemical events, to limit the reperfusion injury (17, 34, 35). Our data indicate that either chronic hexarelin or GH treatment failed in old rats to increase the production of 6-keto-$PGF_{1\alpha}$.by the cardiac endothelium. These negative findings are also consistent with the inability of either treatment to alter the vasopressor activity of angiotensin II: the dose-response curves of this peptide on coronary perfusion pressure during the pre-schemic period were similar in the hearts of the three experimental groups. In whole, these data, in contrast with those obtained in hearts from GH-deficient young-adult rats, where the impairment of endothelial-dependent relaxant function was counteracted by the hormonal treatments (4, 5), indicate that the latter in old rats do not improve the ability of the coronary vascular endothelium to modulate the effect of vasoconstrictors (36).

2.4.Conclusion of Example 2.

The present findings clearly indicate that hexarelin, very likely through a mechanism divorced from its GH-releasing effect, strikingly reduces the reperfusion injury in isolated hearts from senescent rats. The protective effect of hexarelin, which under our experimental conditions, overrides that exhibited by GH, opens new perspectives in the therapy of postischemic heart dysfunction in the elderly. This subject is of increasing interest since the aged population is continuously growing and is becoming one of the major target of pharmacology; moreover, cardiac diseases are the first cause of mortality after 65 years of age (37).

The ability of hexarelin, a recently synthetized hexapeptide with a strong growth hormone (GH)-releasing activity, or of GH itself to display a

protectant activity against post-ischemic ventricular dysfunction in senescent hearts was studied in 24-month-old male rats. Heart preparations from control (saline-treated) senescent rats, subjected to moderate ischemia (stunning), showed at reperfusion: 1) a low recovery of post-ischemic left ventricular developed pressure (LVDP) (37% of the pre-schemic values) coupled to a substantial increase in coronary perfusion pressure (CPP) (71% over baseline); 2) a marked increase of creatine kinase (CK) released in the perfusates (6.6 fold increase over pre-schemic values). *Ex vivo* administration of hexarelin (80 μg/kg, bid, sc) for 21 days resulted in a striking heart protection against reperfusion stunning. In fact, the recovery of LVDP at reperfusion was almost complete (90% of the pre-schemic values) and the increase in coronary resistance was minimal. Furthermore, the concentrations of CK in the perfusates were increased only 2 fold with a gradual return toward basal values at the end of reperfusion. The protectant activity of hexarelin was divorced from any detectable alteration of the somatotropic function, as assessed by pituitary GH mRNA and plasma insulin-like growth factor I levels. *Ex vivo* administration of GH (0.4 μg/g bid, sc) for the same time lapse resulted in only a partial protectant activity: 55% of LVDP recovery; 65% increase of coronary resistance; 5.3 fold increase of CK concentrations in heart perfusates upon reperfusion. Evaluation of the rate of release of 6-keto-$PGF_{1\alpha}$, the stable metabolite of prostacyclin, in heart perfusates and assessment of the vasopressor activity of angiotensin II on the coronary vasculature, did not show any change in these parameters among the three experimental groups. Collectively these data indicate that hexarelin displays a strong heart protectant activity against stunning injury in senescent rats. The protection afforded by the peptide is likely due to a direct cardiotropic action and is far greater than that of GH. Either compound does not appear capable to interfere with the endothelium-dependent relaxant mechanism.

Table 3 Body and heart weights and markers of somatotropic function of 24-month-old male rats treated with hexarelin (HEXA) or growth hormone (GH).

Treatment		Body weight (g)	Heart weight (mg)	Heart weight / Body weight (mg/g)	Pituitary GH mRNA (%)	Plasma IGF-1 (ng/ml)
SALINE (1 ml/kg)	(10)	862 ± 74	2120 ± 175	2.46	100	67.6 ± 11
HEXA (80 µg/kg)	(9)	834 ± 57	2080 ± 218	2.49	+3.4 ± 3.1	70.4 ± 9
GH (0.4 µg/g)	(6)	855 ± 71	2214 ± 165	2.59	-1.0 ± 2.1	73.2 ± 8

Data are mean values ± standard error of the mean. In brackets the number of rats. Drugs were given subcutaneously twice a day for 21 days.

Final conclusion

The findings in these studies thus give evidences for the use of growth hormone (GH) secretagogue compound or GH and Adrenocorticotropic hormone (ACTH) secretagogue compound for the manufacture of a medicament for treating cardiac failure or related vascular dysfunction, against myocardial ischemia and repurfusion events, especially as heart protective agent for patients with heart infarct.

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CLAIMS

1. Use of growth hormone (GH) secretagogue compound, Growth hormone releasing peptide-like (GHRP-like) compound or GH and Adrenocorticotropic hormone (ACTH) secretagogue compound for the manufacture of a medicament for treating cardiac failure or related vascular dysfunction.

2. Use according to claim 1 in which the compound is functioning via the Growth hormone releasing peptide (GHRP) receptor.

3. Use according to any of preceding claims in which the compound is a peptide.

4. Use according to claim 3 in which the compound is a low molecular weight peptide.

5. Use according to claim 4 in which the compound is hexarelin.

6. Use according to any of claims 1 to 5 in which the medicament is used for treatment of impaired cardiac function.

7. Use according to any of claims 1 to 5 in which the medicament is used for treatment of cardiac failure or related vascular dysfunction in GH-deficiency patients.

8. Use according to any of claims 1 to 5 in which the medicament is used for lowering blood pressure.

9. Use according to claim 8 in which the medicament is used for lowering an increased blood pressure.

10. Use according to any of claims 1 to 5 in which the medicament is used for treatment of impaired left ventricular pressure.

11. Use according to any of claims 1 to 5 in which the medicament is used for increase of cardiac output.

12. Use according to any of claims 1-5 in which the medicament is used for prevention of cardiac failure or related vascular dysfunction.

13. Method of treating a mammal with cardiac failure or related vascular dysfunction comprising administration an effective amount of growth hormone (GH) secretagogue compound or GH and Adrenocorticotropic hormone (ACTH) secretagogue compound.

14. Method of treating a mammal for myocardial infarction comprising administration an effective amount of growth hormone (GH) secretagogue compound or GH and Adrenocorticotropic hormone (ACTH) secretagogue compound.

15. Prevention of a mammal for myocardial infarction comprising administration of an effective amount of growth hormone (GH) secretagogue compound or GH and Adrenocorticotropic hormone (ACTH) secretagogue compound.

16. Protection against post-ischemic ventricular dysfunction in a mammal comprising administration of an effective amount of growth hormone (GH) secretagogue compound or GH and Adrenocorticotropic hormone (ACTH) secretagogue compound.

17. Prevention of reperfusion events in a mammal comprising administration of an effective amount of growth hormone (GH) secretagogue compound or GH and Adrenocorticotropic hormone (ACTH) secretagogue compound.

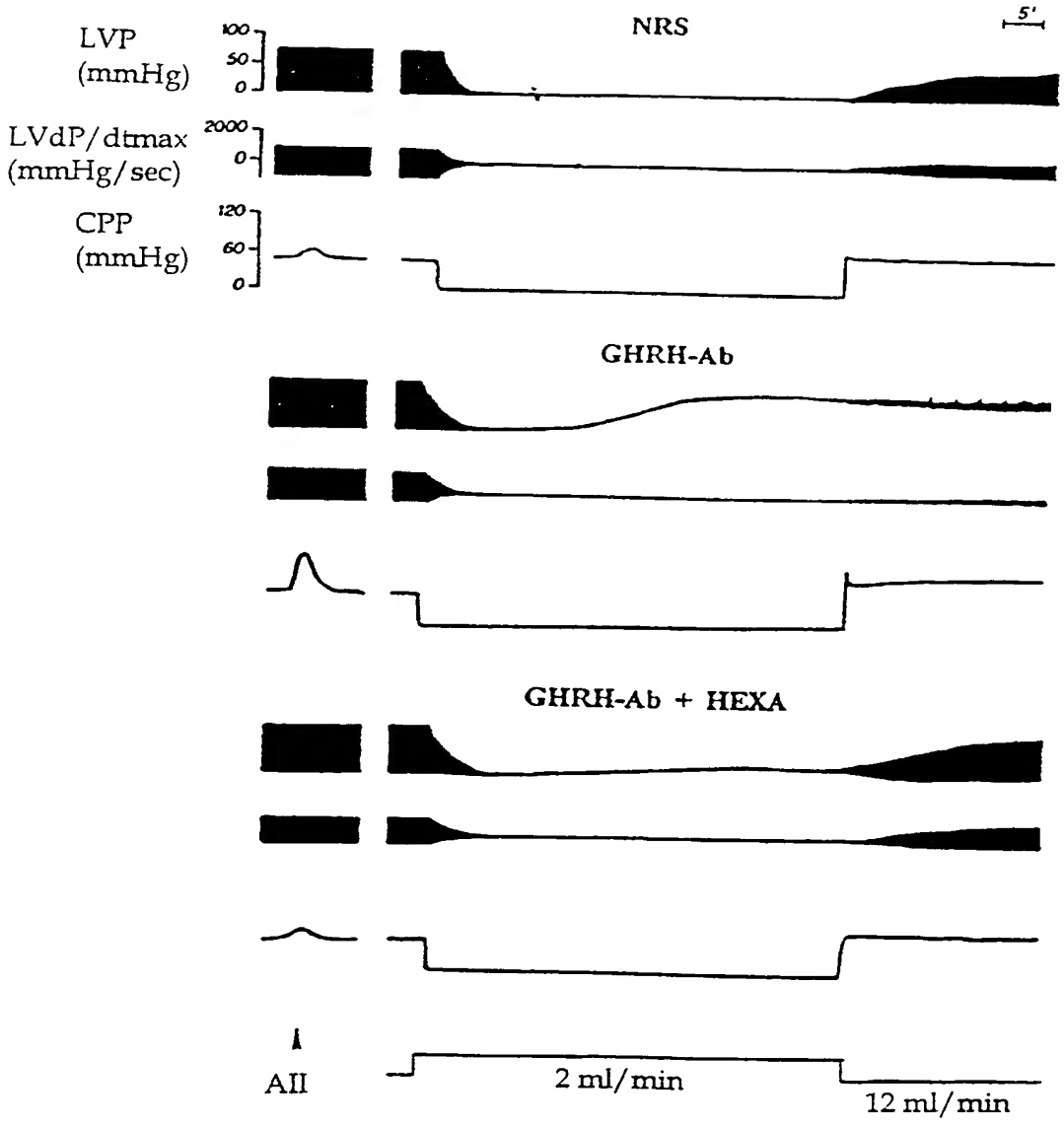

Fig. 1

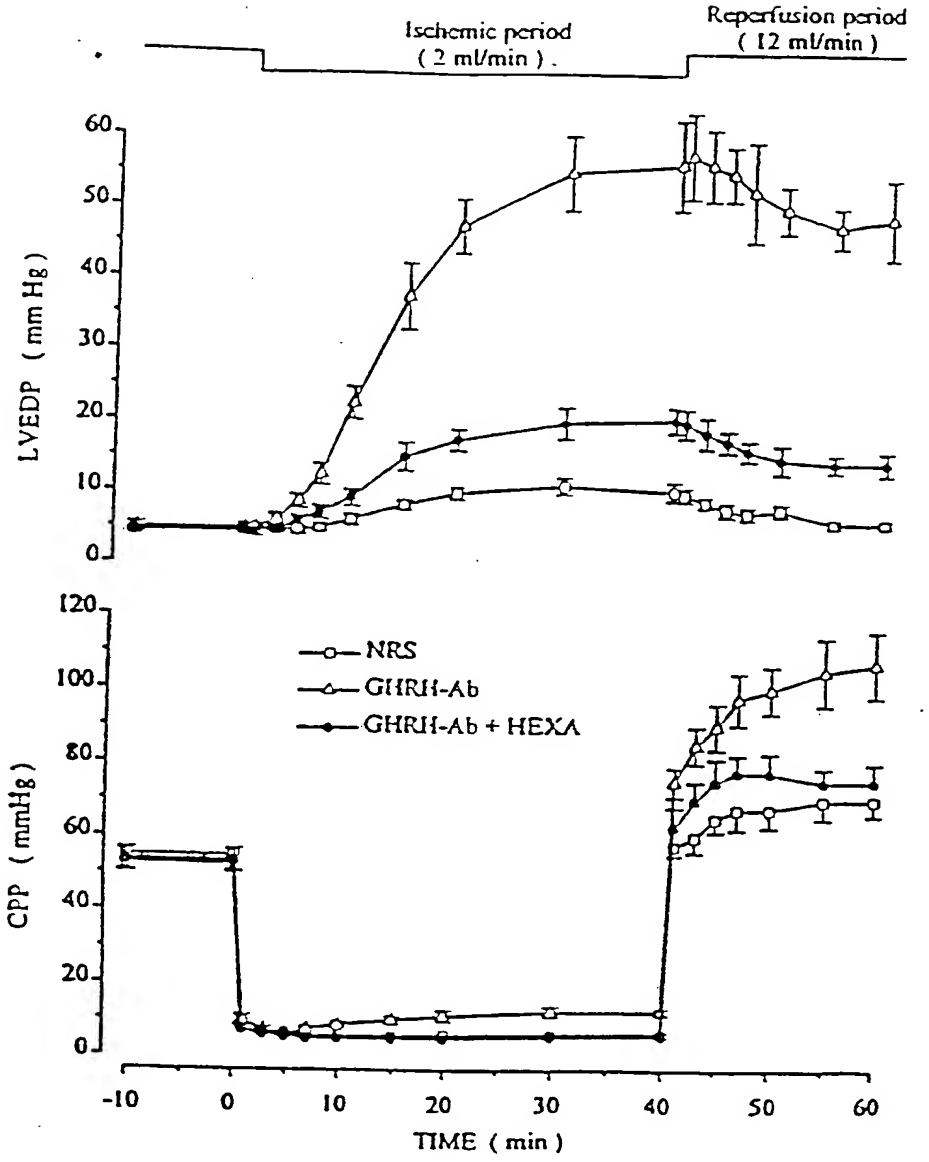
Ischemic period
(2 ml/min)
Reperfusion period
(12 ml/min)
LVEDP (mm Hg)
60
50
40
30
20
10
0
CPP (mmHg)
120
100
80
60
40
20
0
NRS
GHRH-Ab
GHRH-Ab + HEXA
-10
0
10
20
30
40
50
60
TIME (min)

Fig. 2

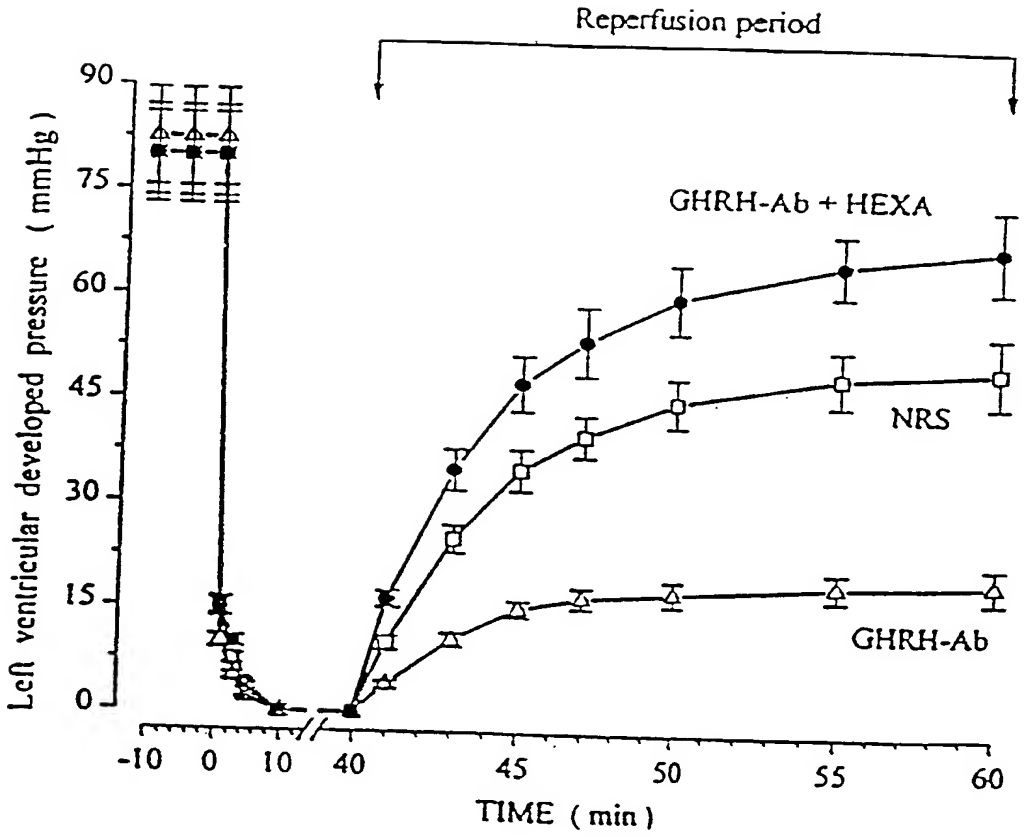

Fig. 3

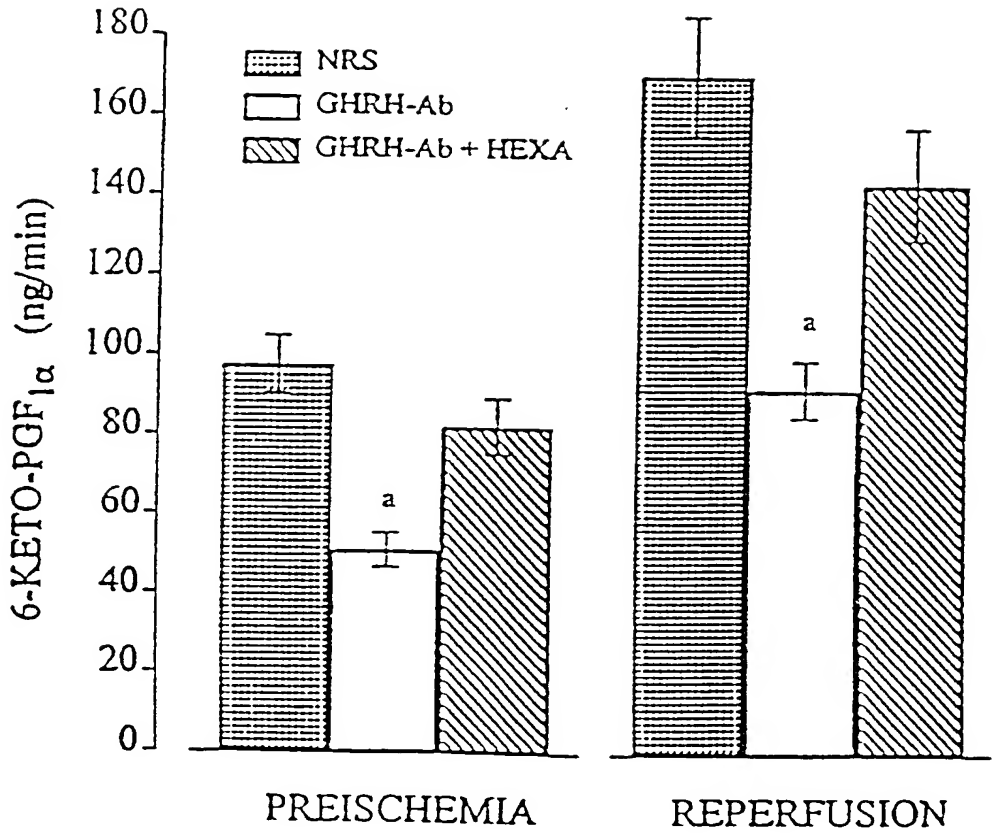
180
160
140
120
100
80
60
40
20
0
6-KETO-PGF1α (ng/min)
NRS
GHRH-Ab
GHRH-Ab + HEXA
a
a
PREISCHEMIA
REPERFUSION

Fig. 4

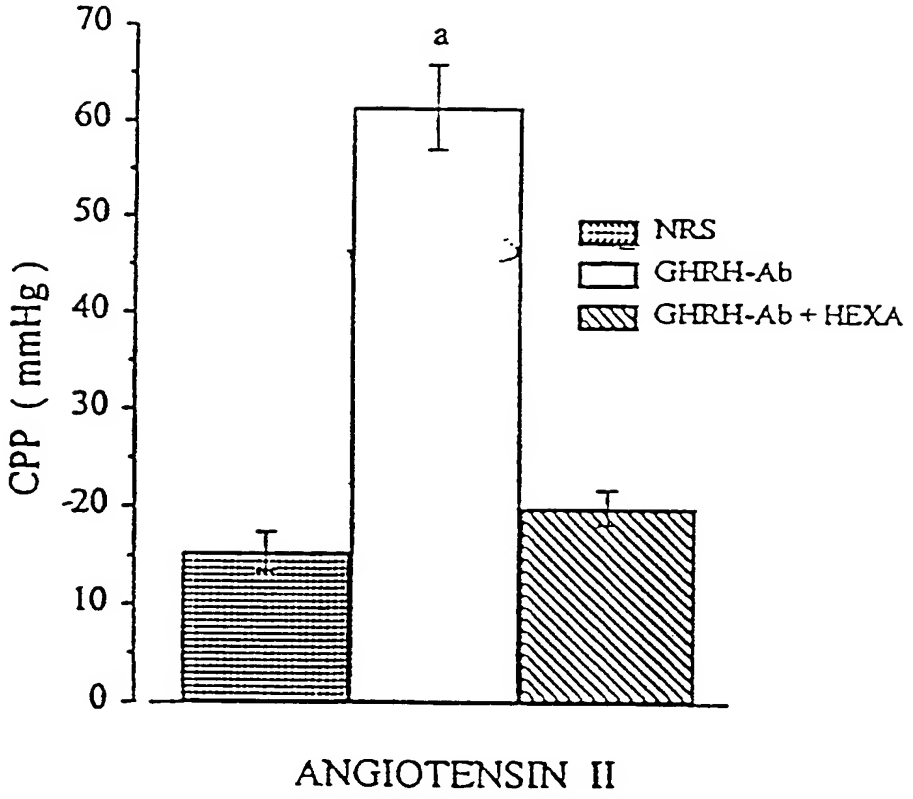
70
60
50
40
30
20
10
0
CPP (mmHg)
a
NRS
GHRH-Ab
GHRH-Ab + HEXA
ANGIOTENSIN II

Fig. 5

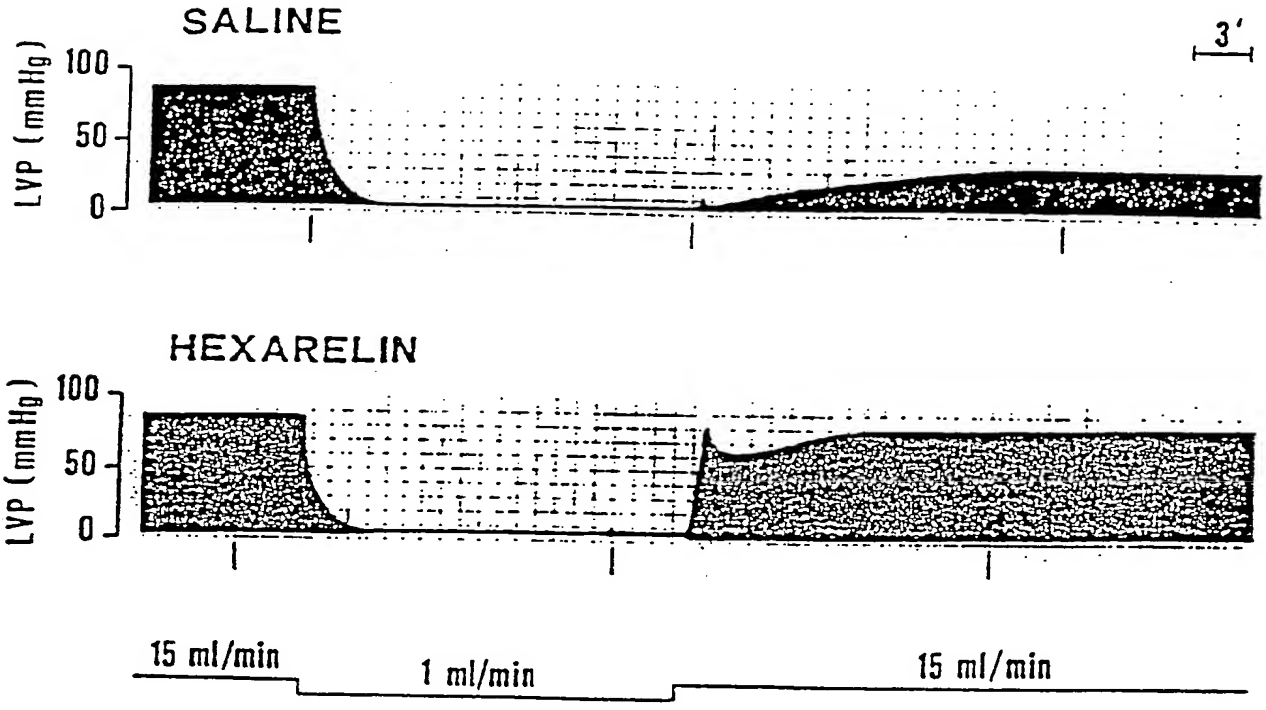
SALINE
3'
LVP (mmHg)
100
50
0
HEXARELIN
LVP (mmHg)
100
50
0
15 ml/min
1 ml/min
15 ml/min

Fig. 6

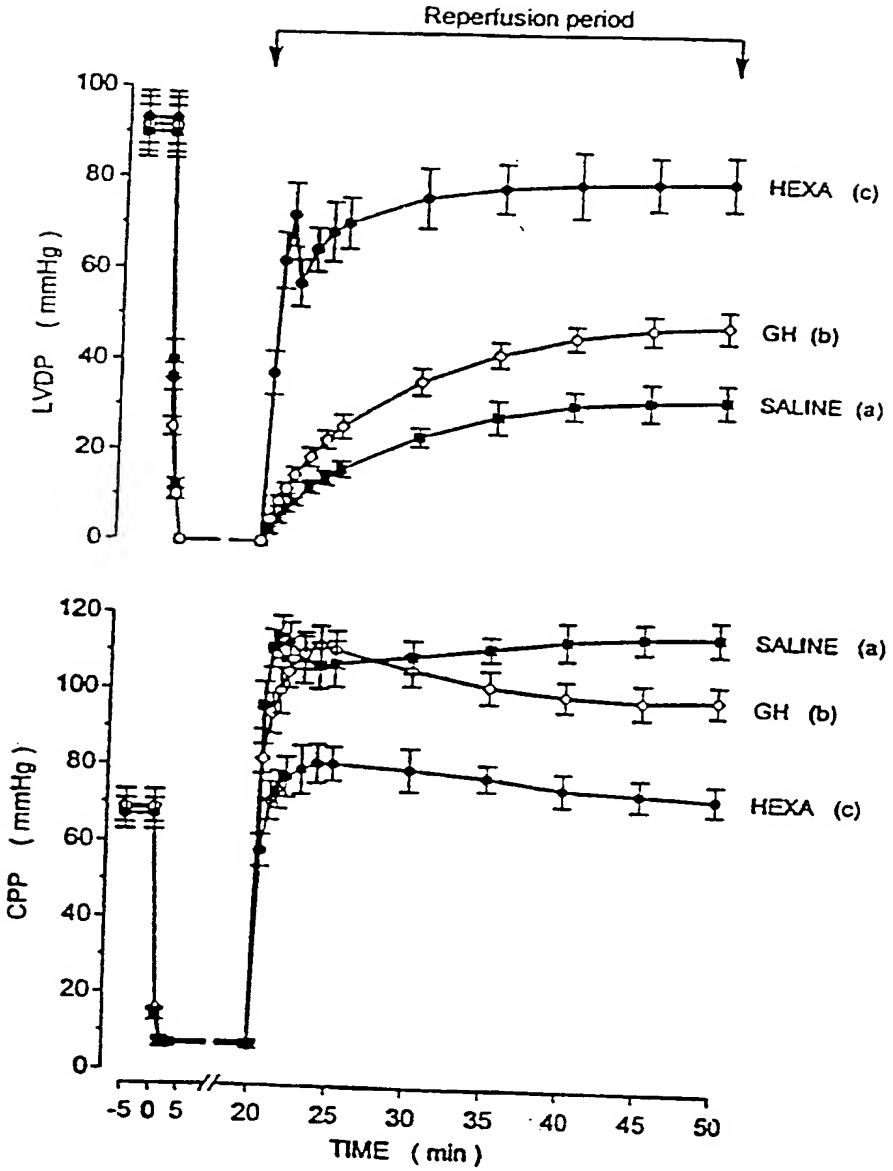
Reperfusion period
LVDP (mmHg)
100
80
60
40
20
0
HEXA (c)
GH (b)
SALINE (a)
CPP (mmHg)
120
100
80
60
40
20
0
SALINE (a)
GH (b)
HEXA (c)
-5 0 5 20 25 30 35 40 45 50
TIME (min)

Fig. 7

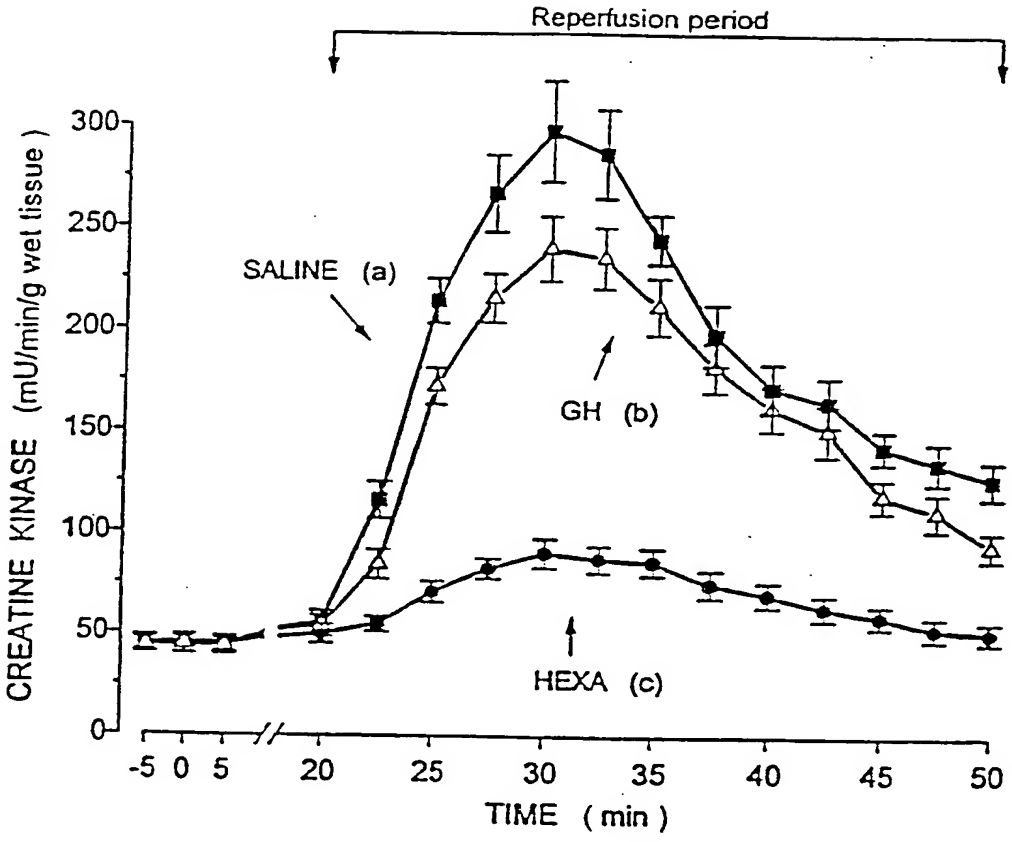
Reperfusion period
CREATINE KINASE (mU/min/g wet tissue)
300
250
200
150
100
50
0
SALINE (a)
GH (b)
HEXA (c)
-5 0 5
20
25
30
35
40
45
50
TIME (min)

Fig. 8

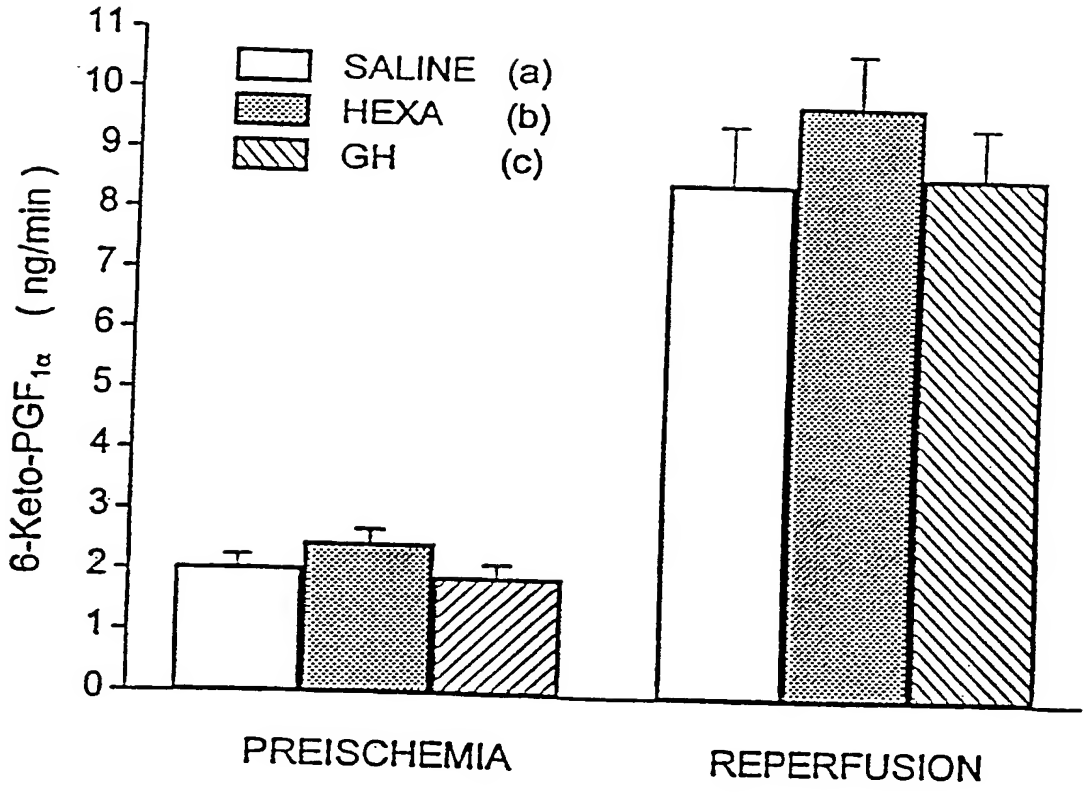
11
10
9
8
7
6
5
4
3
2
1
0
6-Keto-PGF1α (ng/min)
SALINE (a)
HEXA (b)
GH (c)
PREISCHEMIA
REPERFUSION

Fig. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/01957

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 38/08
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EMBASE, MEDLINE, WPI, CLAIMS, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Dialog Information Service, file 155, Medline, Dialog accession No. 07875369, Medline accession no. 94171980, Ghigo E. et al: "Growth hormone-releasing activity of hexarelin, a new synthetic hexapeptide, after intravenous, subcutaneous, intranasal, and oral administration in man", J Clin Endocrinol Metab (UNITED STATES) Mar 1994, 78 (3) p693-8	1-17
Y	WO 9207578 A1 (GENENTECH, INC.), 14 May 1992 (14.05.92), page 4, line 23 - line 31; page 5, line 2 - line 18, claims	1-17

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"B" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 March 1998	24 -03- 1998
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Anneli Jönsson Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/01957

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Dialog Information Service, file 155, Medline, Dialog accession no. 08847887, Medline accession no. 97028762, Kaaja R. et al: "ACTH and growth hormone in myocardial LDH adaptation to hypoxia in rats", Basic Res Cardiol (GERMANY) Jul-Aug1996, 91 (4) P269-74	1-17
	--	
A	Dialog Information Service, file 155, Medline, Dialog accession no. 09010239, Medline accession no. 97076752, Watanabe M. et al: "Cardiovascular effects of intracerebroventricularly administered growth hormone-releasing factor in spontaneously hypertensive rat", Clin Exp Pharmacol Physiol Suppl (AUSTRALIA) 1995, 1 pS58-9	1-17
	--	
A	Dialog Information Service, file 155, Medline, Dialog accession no. 07036466, Medline accession no. 90350126, Macia RA et al: "Hypotension induced by growth-hormone-releasing peptide is mediated by mast cell serotinin release in the rat", Toxicol Appl Pharmacol (UNITED STATES) Jul 1990, 104 (3) p403-10	1-17
	--	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/01957

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 13-17
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Claims 13-17 are directed to method of treatment of the human or animal body by therapy methods practised on the human or animal body/Rule 39.1(iv). Nevertheless, a search has been executed for the claims. The search has been based on the alleged effects of the compounds.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/03/98

International application No.
PCT/SE 97/01957

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9207578 A1	14/05/92	AT	133073 T	15/02/96
		AU	661463 B	27/07/95
		AU	8941291 A	26/05/92
		CA	2092718 A	26/04/92
		DE	69116565 D,T	01/08/96
		EP	0554381 A,B	11/08/93
		SE	0554381 T3	
		ES	2084190 T	01/05/96
		JP	6503320 T	14/04/94
		US	5370870 A	06/12/94

Form PCT/ISA/210 (patent family annex) (July 1992)